Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback

*Gasterosteus aculeatus*

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Abstract

The androgen 11-ketotestosterone (11-KT) plays an important role in reproductive physiology and behaviour in male teleosts. In the three-spined stickleback, *Gasterosteus aculeatus*, the plasma concentrations of 11-KT are related to the breeding status of the fish. Sticklebacks are relatively small (generally less than 1 g) and in order to obtain sufficient plasma for assay of 11-KT, it has been necessary in the past to sacrifice the fish. In this paper, we report on the development of a non-invasive procedure for measuring 11-KT, cortisol and androstenedione (Ad) in the three-spined stickleback. Validation of the procedure included the demonstration that the rate of release of steroids into the water was correlated to their plasma concentrations. Ten males that were kept at a low temperature and short photoperiod were moved to high temperature and long photoperiod to initiate spermatogenesis and breeding. Every two to four days, for a total of 53 days, males were removed and placed in a beaker containing 50-ml water for 30 min. The water was then processed by solid phase extraction for radioimmunoassay. Males were presented with females on days 13/14, 18/19 and 44/45. 11-KT was originally undetectable but built up gradually to reach an average release rate of between 1 and 2.5 ng/g/h between days 16 and 45 and then started to decline (but non-significantly). Ad release reached a plateau of 1 ng/g/h about day 20. However, from days 44/45 to 51, there was a highly significant rise in the rate of release of Ad to 5 ng/g/h. On days 44/45, five of the males mated successfully and five did not. However, there were no significant differences in 11-KT or Ad release rates between the two groups. Cortisol release rates fluctuated with no pattern throughout the study. The results show that it is possible to make measurements on sex and stress steroid production in sticklebacks without recourse to anaesthesia, bleeding or sacrificing the fish. The procedure is potentially a powerful tool for the study of the link between steroids and behaviour in this useful sentinel species.

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1. Introduction

Androgens are key factors for reproductive function in all male vertebrates and usually stimulate the development of secondary sexual characteristics and reproductive behaviour. Testosterone (T) is the major androgen in tetrapod males, but in many male teleosts, including the three-spined stickleback (*Gasterosteus aculeatus*) (Mayer et al., 1990; Borg et al., 1993; Borg, 1994; Borg and Mayer, 1995), the most important androgen appears to be 11-ketotestosterone (11-KT).

Stickleback reproductive behaviour has been studied for over a century and is well characterised. The three phases undertaken by the male stickleback during the reproductive cycle are: nest-building—the male establishes a territory and builds a nest by glueing together plant material; courtship—characterised by a zigzag dance of the male towards the female to attract her to the nest for spawning; and parental care—the male being the one to take care of the eggs. The associated circulating androgen levels have been determined for various stages of the reproductive cycle...
(Mayer et al., 1990; Borg and Mayer, 1995; Páll et al., 2002, 2005). Plasma 11-KT concentrations peak sharply in the male three-spined stickleback between May and July—the height of the breeding season (Mayer et al., 1990). The same observation (a peak in the breeding season) has been made in many other species (Borg, 1994). These studies have yielded important data on stickleback endocrinology. However, in order to obtain the data, many fish have had to be sacrificed—as the bleeding of such small fish (ca. 1 g) is a destructive process (i.e. only one measurement can be made on each individual).

Recently, there has been great interest in the measurement of steroid concentrations in water, rather than in blood plasma (Oliveira et al., 1999; Scott et al., 2001; Scott and Ellis, in press). Stacey et al. (1989) were the first to show that 17,20β-dihydroxyprog-4-en-3-one (17,20β-P), the teleost ‘oocyte maturation-inducing steroid’, was released in relatively large amounts by ovulating female goldfish (Carassius auratus) and that the pattern of release of the steroid matched its pattern of secretion in the plasma. Subsequent studies (see review by Scott and Ellis, in press) have shown that male and female fish of many species release into the water a wide range of sex- and stress-related steroids in free, glucuronidated and sulphated forms.

The advantages of measuring steroids in water as opposed to plasma are: minimal intervention (i.e. no anaesthetic or bleeding), no need to sacrifice the fish, a reduction in sampling stress, the ability to make repeat measurements on the same fish and the ability to make concurrent behavioural observations (Scott and Ellis, in press). It is very important, however, if such a procedure is to replace plasma measurements, that it is properly validated. This is what we have set out to do for the stickleback. The validation includes establishing what steroids are released into the water by males and females, their extraction efficiency and whether the rates of release of steroids are correlated to their plasma concentrations. This is something that has only been done previously for 17,20β-P in goldfish (Stacey et al., 1989), 17,20β-P in dentex (Dentex dentex; Greenwood et al., 2001) and cortisol in rainbow trout (Oncorhynchus mykiss; Ellis et al., 2004). As a test of the practicality of the procedure (and its ability to yield ‘biologically meaningful’ data) we have followed 11-KT, cortisol and androstenedione (Ad) release in individual male sticklebacks at 2–4 day intervals throughout their breeding season (a total of 53 days).

2. Materials and methods

2.1. Fish maintenance

Laboratory-bred three-spined sticklebacks were used. Their parents were originally obtained from a wild population at a reference site in Dorset (Winterbourne Houghton, borehole water of sound environmental quality). Wild fish from the same reference site were also used for one experiment (when laboratory-bred fish were unavailable). The fish were kept in the laboratory in fresh water (de-chlorinated tap water, UV-treated for sterilisation) at 16 ± 2 °C and under a photoperiod of 12L:12D hours (light:dark) unless otherwise stated. The fish were fed daily with frozen bloodworms and the leftovers were regularly removed. Both male and female fish which had been kept under the above conditions weighed ≥0.9 g and only fish showing no external signs of parasitism (in the case of the wild fish) were used in the experiments.

2.2. Steroid screening

Using radioimmunoassay (RIA), we screened for the presence of 12 steroids in the water: 11-KT, Ad, T, cortisol, 17-hydroxyprog-4-en-3,20-dione (17-P), 11-deoxy cortisol (S), 11β-hydroxy-androstenedione (11β-OH-Ad), 5α-dihydrotestosterone (5α-DHT), 17,20x-dihydroxyprog-4-en-3-one (17,20x-P), 17,20β,21-trihydroxyprog-4-en-3-one (20β-S) and 17β-oestradiol (E2).

Four groups of fish (n = 5) were selected: fully reproductive territorial males (wild origin), non-breeding males kept under winter conditions (6L:18D and 10 ± 2 °C; laboratory bred); gravid females (with distended bellies; laboratory bred); and immature females (laboratory bred). All fish were confined individually for 30 min in a 150 ml glass beaker with 50 ml of fresh water (same source as used for aquaria). The fish were returned to their respective tanks and the water samples stored temporarily (up to 1 h) in 50-ml polypropylene tubes at 4 °C. The steroids were then extracted with solid phase extraction (SPE) cartridges (Sep-pak® Plus C18, Waters Ltd., UK) and prepared for RIA as described below. In order to streamline the screening procedure, half of the extract from each fish within each group was pooled and 100 μl aliquots of each of these pools were distributed to 24 assay tubes (two for assay of each steroid). Assay tubes were also prepared, in duplicate, that contained 100 μl of either 0 pg, 10 pg or 100 pg of each of the steroids that were to be screened. To all these tubes was added 100 μl buffer containing 6000 dpm of tritiated steroid and an appropriate amount of antibody to bind 50% of the labelled steroid in the absence of any unlabelled steroid. The tubes were incubated overnight at 4 °C and then separated and counted with dextran-coated charcoal as described previously (Scott et al., 1980). For those steroids where there was evidence of large differences between the pools (11-KT and Ad), the original extracts were reassayed individually with full (and thus more accurate) standard curves.

2.3. Extraction efficiency and steroid stability

A 3 l beaker was filled with 2 l of fresh water (the same source as for the aquaria). A known amount of 11-KT, Ad and cortisol was added into the beaker to achieve a final concentration of 5 ng/50 ml for all steroids. Samples of 30 ml were transferred into 30 polypropylene centrifuge tubes. Five were extracted directly, five were frozen and extracted the day after, whilst the others were stored at 4 °C. These were extracted after 1, 2, 6 and 24 h (five samples for each time point). After addition of RIA buffer, all samples were frozen until RIA.

2.4. Time release of steroids into water

Reproductively mature (wild source) male sticklebacks were moved four times between beakers containing 50 ml fresh water with a stay of 30 min in each. The fish were then returned to their tanks and the water samples extracted and prepared for RIA.

2.5. Identity of steroids

Nine laboratory-bred males which had been held in individual tanks under summer conditions (16L:8D and 16 ± 2 °C) for 2 months were moved into individual beakers with 50 ml of fresh water for 60 min. The water samples were then pooled, extracted and dried down. The extract was then reconstituted, filtered and loaded on to and run on an analytical reverse-phase HPLC column (Dynamax Microsorb; 5 μm octadeccylsilane; 4.6 mm × 250 mm; fitted with a 15 mm guard module; Rainin Instrument,
Laboratory-bred males (n = 24), all coming from the same stock tank, were used in this experiment. Each male was placed in a 150 ml beaker with 50 ml of fresh water for 30 min prior to euthanasia in MS222. The blood was collected via caudal severance using microhaematocrit capillary tubes. The water samples were extracted as described above. The blood samples were centrifuged at 13,000 rpm for 5 min at 4°C and stored at −20°C. After thawing, they were re-centrifuged at 13,000 rpm for 3 min at 4°C and 5 μl was transferred into a 1.5 ml Eppendorf, to which was added 100 μl of distilled water and 1 ml ethyl acetate. The tubes were vortexed for 10 s, then centrifuged at 13,000 rpm for 3 min at 4°C. The bottom of the Eppendorf was placed briefly into liquid nitrogen to freeze the aqueous phase and the organic phase was poured into a glass tube (12 × 75 mm). The extraction was then repeated. The combined ethyl acetate extracts were dried under a nitrogen stream at 45°C then redissolved in 500 μl of RIA buffer and frozen for subsequent assay.

2.6. Correlation between plasma and water steroid levels

Laboratory-bred males were kept under winter conditions (i.e. 6 L:18D photoperiod and a temperature of 10 ± 0.5°C) for 2 months prior to the onset of the experiment, whilst female fish were kept under breeding conditions (16L:8D, 18°C) for the same period. We have repeatedly noted that the time needed for female sticklebacks in captivity to complete vitellogenesis and enter spawning is considerably longer that the time needed by the average male.

On day 1, 10 males were moved to individual 150 ml beakers with 50 ml of fresh water for 30 min. They were then weighed, measured and transferred to 30 l individual tanks containing 20 l of fresh water to acclimate. Partitions were positioned between all the tanks so that there was no visual contact between the fish. Gradually, the temperature and the photoperiod were increased to 12.5 ± 0.5°C, 12L:12D, 100–200 lux on day 0. Finally, on day 1, the temperature was increased to 18 ± 0.5°C and the photoperiod to 16L:8D, 1000–1200 lux. On the same day, water samples were collected as described above. However, this time, the beaker was placed inside the tank (Fig. 1) in an attempt to minimise potential confinement stress.

On day 6, the males were provided with nest material and gravel. At the same time, the partitions between two adjacent tanks were removed for 1 h in the morning and 1 h in the afternoon, in order to motivate the males to build a nest (we have observed in previous trials that the visual cue of a rival male stimulates building behaviour). From day 1 to day 20 the water collection procedure was repeated every two days, between 9 and 10 a.m. and before feeding. Another set of water samples started from day 27 to day 51. The water collection took place every four days until mating was attempted on day 44/45 and then every two days at the same time of the day.

Mating was attempted on days 13, 18 and 44 for five of the males and days 14, 19 and 45 for the remaining five. On mating days, water samples were collected as described above from the males before their encounter. Each male was then returned to its tank, allowed to acclimate for 15 min and presented with a female for 15 min. Courtship behaviour and spawning were recorded. After removal of the female, the male was left for 15 min and then transferred to a beaker for a second sample. Five males mated successfully on the final occasion (days 44/45). In order not to disturb the nesting activity of these males, the partitions were left in place for the remainder of the sampling period (i.e. the fish were no longer given daily visual contact with each other).

In all water samples, 11-KT, Ad and cortisol (to check if the frequency of sampling increased the levels of stress), as well as T for a small section of the cycle, were measured.

2.8. Radioimmunoassay and extraction procedure

3H-labelled steroids were purchased directly from Amersham Biosciences, UK (E2, 17-P, T, 11-KT, cortisol, Ad, 5α-DHT) or American Radiochemicals (17,21-P); or synthesised from commercially available precursors (17,20β-P, 11β-OH-Ad, 17,20α-P and 20β-S) (Scott et al., 2005). Ten antisera came from previously recorded sources (see Lower et al., 2004) where the same sort of study was carried out. Anti-5α-DHT was purchased from Biogenesis Ltd., Poole, UK and anti-11β-OH-Ad was a gift from Dr. Schulz (Schulz, 1985). The RIA procedure has been described previously (Scott et al., 1984; Ellis et al., 2004).

For extraction of steroids, 50 ml water samples were pumped under vacuum at a rate of ca. 3 ml/min through primed SPE cartridges containing octadecylsilsane (Sep-pak Plus, Waters Ltd., Watford, UK). The reservoirs were formed from 60 ml syringes and samples were not pre-filtered. Priming involved 5 ml methanol followed by 5 ml distilled water. After the samples had been pulled through, the cartridges were washed with 5 ml distilled water followed by 20 ml air (to remove as much moisture as possible). The steroids were eluted with 5 ml ethyl acetate. This was collected in a glass tube and evaporated at 45°C under a stream of nitrogen. The steroids were redissolved in 1 ml of RIA buffer (0.5 M phosphate buffer containing 0.2% sodium azide, 0.8% sodium chloride, 0.03% EDTA and 0.01% sodium azide) and stored at −20°C until required for RIA.

2.9. Statistical analysis

Paired comparisons were carried out using MINITAB statistical software (version 12). An Anderson–Darling test was performed to test the data distribution. The data were not normally distributed and thus were subject to non-parametric analyses, using the two-tailed Mann–Whitney U test for comparison between two groups, at 95% significance level. For determining the significance of the correlation between plasma and water steroid levels, the Spearman correlation test was applied. For comparing changes in steroid release over time between 39 and 51 days in the seasonal cycle experiment, data were logarithmically transformed and analysed with SAS General Linear Model (GLM) and post-hoc Duncan’s test.

3. Results

3.1. Steroid screen

There was no evidence for the release (detection level of 0.2 ng/fish/h) of 17,20α-P, 17,20β-P, 20β-S or E2 by any of the fish (Fig. 2). There was a measurable rate of release of material that cross-reacted in the assays for 17-P and S, but without clear distinction between the groups. There was a higher rate of release of Ad, cortisol, 11β-OH-Ad, 5α-DHT and T in mature fish than in immature fish. However, there was no apparent difference between the sexes. 11-KT, on the other hand, was released only by mature males and in the highest amounts of any of the steroids (>2 ng/fish/h).
Analysis of 11-KT and Ad release in individual fish (as indicated by error bars in Fig. 2) showed that the territorial males produced significantly more 11-KT than any of the other groups ($P < 0.05$). Ad was detected in much higher levels in both territorial males and gravid females than in quiescent males and immature females ($P < 0.05$). However, no significant difference in Ad release was found between the territorial males and the gravid females.

3.2. Extraction efficiency and steroid stability

The recovery of all steroids was between 95% and 105%. After 1 h at 4°C, there was an 8% drop in 11-KT, 6% in Ad and 2% in cortisol (Table 1). However, after 24 h, the 11-KT had still only dropped 8%, Ad 14% and cortisol 11%. These were similar to the losses found when freezing the samples.

3.3. Time release of steroids in water

The sequential transfer experiment showed that 11-KT and Ad release decreased by 60% and 70%, respectively, between the first and the fourth sampling periods (Fig. 3). Cortisol release, on the other hand, spiked sharply during the third sampling period and then returned to its original level of around 1 ng/fish/h (the fish were not weighed in this experiment).

3.4. Identity of steroids

The HPLC separation of water extracts from breeding males, followed by RIA of the fractions, showed that 11-KT, Ad and cortisol had sharp peaks of activity corresponding to their expected elution positions—with small amounts of diffuse activity in other fractions for Ad and cortisol but a distinct second peak (11% of activity; identity unknown) for 11-ketotestosterone (11-KT) and androstenedione (Ad), the steroid levels were assayed in individual fish (the bars representing SE). All other steroids were measured in pooled extracts. $n = 5$ fish for each group.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Territorial males</th>
<th>Quiescent males</th>
<th>Gravid females</th>
<th>Immature females</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-KT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,20β-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,20α-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11β-OH-Ad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-DHT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,20β,21-trihydroxyprog-4-en-3-one</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20β-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Profile of steroids released into the water by three-spined sticklebacks at different reproductive stages. The line represents 0.20 ng/fish/h (based on the lowest standard concentration of steroid in the initial scan). T, testosterone; 17-P, 17-hydroxypregn-4-ene-3,20-dione; S, 11-deoxycortisol; 11β-OH-Ad, 11β-hydroxy-androstenedione; 5α-DHT, 5α-dihydrotestosterone; 17,20α-P, 17,20α-dihydroxyprog-4-en-3-one; 17,20β-P, 17,20β-dihydroxyprog-4-en-3-one; 20β-S, 17,20β,21-trihydroxyprog-4-en-3-one; E2, 17β-oestradiol. For 11-ketotestosterone (11-KT) and androstenedione (Ad), the steroid levels were assayed in individual fish (the bars representing SE). All other steroids were measured in pooled extracts. $n = 5$ fish for each group.

Table 1

<table>
<thead>
<tr>
<th>Extraction time (h)</th>
<th>11-KT (±SE)</th>
<th>Ad (±SE)</th>
<th>Cortisol (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 3.8</td>
<td>100 ± 1.5</td>
<td>100 ± 13.3</td>
</tr>
<tr>
<td>1</td>
<td>91.6 ± 5.3</td>
<td>94.4 ± 4.0</td>
<td>98.2 ± 9.1</td>
</tr>
<tr>
<td>2</td>
<td>90.2 ± 3.7</td>
<td>89.7 ± 7.0</td>
<td>93.4 ± 9.4</td>
</tr>
<tr>
<td>6</td>
<td>89.3 ± 2.9</td>
<td>91.8 ± 4.2</td>
<td>90.4 ± 1.05</td>
</tr>
<tr>
<td>24</td>
<td>91.9 ± 2.3</td>
<td>86.4 ± 4.4</td>
<td>89.8 ± 3.3</td>
</tr>
<tr>
<td>Frozen samples</td>
<td>88.9 ± 5.8</td>
<td>93.6 ± 3.6</td>
<td>85.5 ± 9.5</td>
</tr>
</tbody>
</table>

The values are percentage of recovery (±SE) of initial (0 h) samples. $n = 5$. 11-KT, 11-ketotestosterone; Ad, androstenedione.
whether the fish were not producing much steroid or whether there had been losses during or after HPLC.

3.5. Correlation between water and plasma levels

There was a highly significant positive linear correlation between plasma and water concentrations of 11-KT ([math]r_s = 0.85, n = 24, P < 0.001) and a significant positive curvilinear correlation between plasma and water concentrations of cortisol ([math]r_s = 0.82, n = 24, P < 0.001) (Fig. 5). There was also a significant correlation between plasma and water concentrations of Ad ([math]r_s = 0.75, n = 24, P < 0.001 for all males; [math]r_s = 0.91, n = 6, P < 0.05 for males with detectable plasma concentrations). However, plasma Ad was below the limits of detection for many of the samples. Using rough estimates (see Fig. 5 for equations), a release rate of 3 ng/g/h corresponds to a plasma concentration of 100 ng/ml for 11-KT, 75 ng/ml for cortisol and only 5 ng/ml for Ad.

3.6. Steroid release rates in male sticklebacks during a complete reproductive cycle

Progressive changes in 11-KT, Ad and cortisol release rates by the 10 male sticklebacks are shown plotted with means ± SE on a linear scale in Fig. 6. Individual 11-KT and Ad release rates between days 7 and 51 (omitting the second samples on days 13/14, 18/19 and 44/45) are shown plotted on a logarithmic scale in Fig. 7. Up until day 5, neither 11-KT nor Ad were detectable. On day 6, nesting material was added to the tanks and, between day 5 and 7, there was a slight increase in the release of both steroids. From day 7 till day 20, 11-KT release increased from 0.26 to about 2.5 ng/g/h and then maintained a rough plateau up until days 44/45, at which time it fell (note the downward trend of quadratic plot in Fig. 7). Ad also increased from day 7 to day 20 and maintained a plateau of ca. 1 ng/g/h until days 44/45, at which time it rose steeply (note upward trend in cubic plot in Fig. 7). Cortisol release could be detected at all sampling times. It fluctuated between 0.20 and 1.00 ng/g/h except on days 18/19 (a weekend) when it increased sharply to 2.16 ± 0.28 ng/g/h (cause unknown). Measurements were made of T release between 34 and 48 days (Fig. 6). The pattern was the same as that of 11-KT but ca. twofold lower.

On days 13/14, 18/19 and 44/45 (matting attempts) one female was added to each tank. On the first two occasions, the rate of release of 11-KT decreased between before addition and after removal of the females from the tank. Ad, however, was little affected and there appeared to be no
long-term consequences for the fish for rates of release of either steroid. No spawning occurred on either of these two dates. At the final mating attempt (day 44/45), five of the males were successful in getting females to spawn in their nests. Nevertheless, it was not possible to statistically demonstrate any differences in 11-KT and Ad release rates between the successful and the unsuccessful males. However, two of the nesting males on day 51 (the two lowest symbols in Fig. 7 a) had 11-KT release rates that had fallen below 0.1 ng/g/h (although were still measurable).

4. Discussion

The steroid-screening experiment revealed that 11-KT is the predominant steroid produced by male sticklebacks and that it is absent in, or at least produced in only very low amounts by females and immature males. It also revealed unexpectedly high release of Ad by both mature males and females. This is a steroid that has received very little attention in the past. However, it is one that is attracting interest because it is released into the water in particular abundance by male and female goldfish where it acts as a pheromone (Sorensen et al., 2005). The release of T by both sexes (again higher in the mature than immature fish) was expected as it is well known that this steroid is ubiquitous in male and female teleosts (Borg, 1994). 17-P and S are known to be released in large amounts by ovulating female goldfish. However, their release rates in the stickleback are relatively low and seem not to be linked to reproductive stage or sex. The antibody for S is known to have a small cross-reaction with cortisol and this might be the reason for its apparent presence in the water. The absence of release of the putative ‘maturation-inducing steroids’ 17,20β-P, 20β-S and 17,20α-P by the mature females could be because they were not at the appropriate stage of maturation (i.e. periovulation). However, 17,20β-P was also not detected in previous studies (Borg et al., 1989; Mayer et al., 1990). This suggests that C21 steroids do not play a major part in either spermiation, male behaviour or male pheromone signalling in this species. The rate of release of E2 by mature females in the present study was very low in comparison to T. Based on the fact that 11-KT was only found in the males, we were not expecting to find 11β-OH-Ad in females as well as males. However, this steroid could originate from the interrenal gland–as its pattern of secretion (by sex and stage) was similar to that of cortisol. This contention is supported by the fact that 11β-OH-Ad
appears to be present in plasma (at concentrations up to 18 ng/ml) throughout the reproductive cycle in male sticklebacks and does not appear to be linked to changes in 11-KT concentrations (Mayer et al., 1990). Furthermore, 11β-OH-Ad concentrations were not affected by castration whilst those of 11-KT were (Mayer et al., 1990). A factor that has to be taken into account in all studies involving immunoassay is cross-reaction (e.g. the RIA for S mentioned above). This probably accounts for a varying proportion of the measured activity of all of the steroids. The HPLC scan of a water extract revealed that, even for 11-KT, there was an unknown cross-reacting steroid that contributed 11% of the total measurable activity. This will need to be investigated further.

Based on experiments in goldfish and rainbow trout (Vermeirssen and Scott, 1996; Sorensen et al., 2000; Ellis et al., 2005), it can be assumed that the steroids that we are measuring are released into the water by diffusion across the gills. The underlying principle of measuring steroids in water rather than plasma is that the release rate is equivalent to plasma concentration. Concerning 11-KT, we have found a convincing linear relationship. Concerning cortisol, we have found a similarly strong relationship, but it is markedly curvilinear. In rainbow trout (Ellis et al., 2004), the water–plasma relationship for cortisol was linear. This could reflect a difference between species. However, in the rainbow trout experiments, the fish were not handled in any way prior to being sacrificed and bled, while in the stickleback experiments, the fish had to be put in a collection beaker for 30 min prior to bleeding. In that time interval, some of the fish could have become very stressed (resulting in very high plasma concentrations), but the extra cortisol in the blood would perhaps not have had time to diffuse into the water (hysteresis effect).

Although there was a linear relationship between release rates and plasma concentrations of Ad, the plasma concentrations were much lower than expected on the basis of its high binding affinity for plasma sex steroid-binding globulin (see Scott et al., 2005). This disparity will need further investigation.

The non-invasive procedure used in this study appears to be very effective for monitoring the main teleost androgen, 11-KT, in the male three-spined stickleback during the nest-building and sexual phases of reproduction. There was no problem with assay sensitivity. In many cases, the extracts had to be diluted 10-fold to fall on the standard curve. This suggests that the confinement time (30 min) could be reduced. However, it is useful to have sufficient extract for re assay, or for assay of other steroids. A longer confinement time is not recommended, as one of our experiments seemed to indicate that both 11-KT and Ad production decreased significantly when the fish were kept in confinement for up to 2 h. Although there could have been other reasons for this drop (e.g. it could have been part of a diurnal cycle), it is more likely to be a ‘handling effect’. It has been frequently reported that, in fish that are bled repeatedly, there is a drop in androgen concentrations in the plasma (Schreck, 1972; Scott and Canario, 1990; Vermeirssen et al., 2000). This has been suggested to be due to a combination of handling stress and blood dilution (Schreck, 1972). Only the first of these is applicable to the present situation and appears to be supported by the sudden increase in cortisol release between 60 and 90 min.

It is not so easy to compare our results directly with those of other who have measured 11-KT in water from other species (Oliveira et al., 2003; Hirschenhauser et al., 2004; Bender et al., 2006; Dzieweczynski et al., 2006; Earley et al., 2006; Rodgers et al., 2006). In all but the last two studies, the release rate was reported of free + glucuronidated + sulphated 11-KT. In the present study, we have only measured the free fraction. Also, only one of the other studies (Dzieweczynski et al., 2006) attempted to compare water release rates with plasma concentrations, but had insufficient data to prove a relationship. Nevertheless, all studies indicate that 11-KT (whether free or conjugated) is easy to measure in water using a wide variety of species.

All above-mentioned studies, plus the present one, use a similar procedure—placing a fish in a beaker or jar for between 30 and 120 min. Its was first used in 1998 by Barata and colleagues (Oliveira et al., 1999) to demonstrate the release of free and sulphated 17,20β-P into the water by male peacock blennies (Salarias pavo; 10 cm length, 500 ml water, 90 min). Although the procedure is mild (compared to anaesthesia and bleeding), it still involves handling and confining the fish and changing its environment. Thus, it is arguable as to what extent the procedure can be used in association with cortisol measurement to study stress (Bender et al., 2006; Earley et al., 2006). The reason we measured cortisol in the present study was to determine whether the sampling procedure was a long-term stressor. However, although the cortisol release rate was not constant, there was no evidence of any pattern and no evidence that the level of release either increased or decreased with time. This adds to the reputation of sticklebacks as being a very ‘tough’ species and resistant to frequent handling. It has been shown previously, for instance, that if a stickleback male was netted out and injected with solvent there was no difference in their courtship behaviour tested before or 1 h after (Pall et al., 2004).

The absence of release of 11-KT by non-breeding males and substantial release of 11-KT by breeding males is consistent with the results of previous studies that have shown plasma concentrations of 11-KT to be undetectable in fish outside the breeding season (<2 ng/ml) and high (43 ng/ml) (Mayer et al., 1990) or sometimes very high (up to 400 ng/ml in some individuals) within the breeding season (Pall et al., 2005, 2002). Since these previous studies involved sacrificing the fish, it was not possible to study changes in the same individual, nor was it practicable to monitor steroid production in more than a few fish at a time over a complete reproductive cycle. With our procedure, we have been able to show that the males start to produce detectable 11-KT about five days after an increase in temperature and photoperiod. It then takes them another 15 days or so
to reach a plateau of 11-KT production which they appear to be able to maintain for many days (at least 25). During this time, all fish were allowed sight of their neighbour for an hour twice daily and were also introduced to a female on three occasions. These procedures form part of our normal husbandry practice and were not conducted as experiments on the fish (i.e. there were no controls). Thus, we can draw no firm conclusions on whether, if the fish had not been allowed to see each other or to interact with females, they would still have shown the same changes in hormone production. We also cannot draw any conclusion as to the necessity of 11-KT for nest-building (Borg and Mayer, 1995; Bell, 2001) other than to point out that the males only commenced nest-building when 11-KT release rates reached ca. 1 ng/g/h.

On the third occasion that the males were given females, five of the males spawned successfully and five did not. Rather than a fall in 11-KT concentrations, there was an immediate (but not statistically significant) rise in both groups. Thenceforward, release rates fell slightly, but not significantly, in both groups. Based on previous studies (Páll et al., 2002, 2005), we expected to find a marked drop in 11-KT release rates in the males that became parents. However, this only happened with two out of five males.

We have shown in the present study that it is possible also to measure T in water in both males and females. It was measured at six sampling times in the males and its pattern of release matched that of 11-KT but at a lower level. Unfortunately, we did not have sufficient plasma to check the relationship between plasma concentrations and water release rates of T. Thus, measurement of this steroid in water from sticklebacks must be considered to be not yet fully validated.

In conclusion, we have developed and fully validated a procedure for non-invasive measurement of 11-KT, Ad and cortisol in male sticklebacks and shown that it is possible to monitor individuals over a complete reproductive cycle without losing a single fish. The procedure has potential use in a wide range of physiological, behavioural and ecotoxicological studies.

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References


